J. Clin. Chem. Clin. Biochem.
Vol. 23, 1985, pp. 129–135

Determination of Uric Acid in Human Serum by Isotope Dilution-Mass Spectrometry

Definitive Methods in Clinical Chemistry, III

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(Received August 8/October 29, 1984)

Summary: A method for the measurement of uric acid in human serum by isotope dilution-mass spectrometry is described. The analytical procedure consists of the following steps:

(1) Addition of [1,3-$^{15}N_2$]uric acid to the serum sample;
(2) ion exchange chromatography on AG1-X2;
(3) formation of the trimethylsilyl derivative;
(4) gas liquid chromatography-mass spectrometry (GC-MS), selected ion monitoring (SIM) at m/z-values 456 and 458;
(5) calculation of the amount of uric acid in the serum sample from the isotope ratio, as measured by GC-MS.

The accuracy of the method is obtained by the use of the highly specific technique of selected ion recording in combination with the exact control of recovery as performed by the isotope dilution procedure. On the basis of the high accuracy of the isotope dilution-mass spectrometry technique, the method presented here may be proposed as a definitive method in clinical chemistry.

The imprecision of the method was estimated by measuring replicates in 18 lyophilised serum pools on different occasions. The coefficient of variation proved to be between 0.6 and 1.1% in the concentration range of 200 to 500 µmol/l. The lower limit of detection (ratio of signal to noise 3 : 1) of SIM was about 10 ng uric acid per sample.

Bestimmung von Harnsäure im menschlichen Serum mit Hilfe der massenspektrometrischen Isotopenverdünnungsanalyse

Definitive Methoden in der Klinischen Chemie, III

Zusammenfassung: Es wird eine Methode zur Messung von Harnsäure in menschlischem Serum mit der massenspektrometrischen Isotopenverdünnungsmethode beschrieben. Das Analysenverfahren umfaßt folgende Schritte:

(1) Zugabe von [1,3-$^{15}N_2$]Harnsäure zu der Serumprobe;
(2) Ionenaustauschchromatographie an AG1-X2;
(3) Bildung des Trimethylsilylderivats;
(4) Gaschromatographie-Massenspektrometrie, massenspezifische Detektion bei den m/z-Werten 456 und 458;
(5) Berechnung der Menge an Harnsäure in der Serumprobe aus dem massenspektrometrisch ermittelten Isotopenverhältnis.

Die Richtigkeit der Methode wird durch die Anwendung der hochspezifischen Technik der massenselektiven Aufzeichnung charakteristischer Ionen in Kombination mit dem Isotopenverdünnungsverfahren erreicht. Auf Grund der hohen Richtigkeit der massenspektrometrischen Isotopenverdünnungsanalyse kann die hier vorgestellte Methode als Definitive Methode in der Klinischen Chemie vorgeschlagen werden.

Die Impräzision wurde durch Mehrfachanalysen von 18 lyophilisierten Serumpools an verschiedenen Tagen ermittelt. Der Variationskoeffizient lag im Bereich von 0,6 bis 1,1% bei einem Konzentrationsbereich von 200 bis 500 µmol/l. Die untere Nachweisgrenze der massenselektiven Detektion (bei einem Signal-zu-Rausch-Verhältnis 3 : 1) beträgt etwa 10 ng Harnsäure pro Probe.

Introduction

Many methods for the determination of uric acid in human serum have been proposed in clinical chemistry. The most commonly used procedures are the colour reaction with phosphotungstic acid (1), the enzymatic reaction with uricase (2, 3) and the combined enzymatic reactions with uricase and catalase (4) as well as with uricase and aldehyde dehydrogenase (5, 6, 7). More recently, procedures which are based on the enzymatic reaction with uricase (8) as well as on the technique of mass fragmentography (9, 10, 11) have been proposed as reference methods.

Vast difference between the results of various routine methods applied to the same serum sample have been observed (12). This fact is also obvious from the different method-dependent target values of external quality control surveys of the Deutsche Gesellschaft für Klinische Chemie. Therefore it became necessary to develop reference and definitive methods in order to provide a reliable basis for comparison and improvement of procedures for the measurement of uric acid. Furthermore, the use of definitive or reference method values as target values in external quality control surveys may be considered as an invaluable aid for improving accuracy in the clinical chemical analysis of uric acid in general. They would help to direct the results of all laboratories more and more towards "true values", not only in external quality control but also in routine work.

Principle of the Method

A definite amount of isotopically labelled uric acid is added to a serum sample containing an unknown amount of the non-labelled substance, both of which are purified by ion exchange chromatography and then converted to the trimethylsilyl derivates. The ratio of non-labelled to labelled substance is measured by combined gas chromatography-mass spectrometry using the selected ion monitoring technique. The unknown amount of uric acid in the serum sample is calculated from the isotope ratio.

Materials and Procedure

Reagents

Uric acid (purity 99.7%) is obtained as a standard reference material from the National Bureau of Standards, Washington, USA. [1,3-$^{15}N_2$]Uric acid (99% ^{15}N) and [2-^{14}C]uric acid, specific radioactivity 2.22 GBq/mmol, are obtained from Amersham Buchler, Braunschweig, FRG. Anion exchange resin (AG1-X2, acetate form, 200–400 mesh) was supplied by BioRad, München, FRG. N-Methyl-N-trimethylsilyl-trifluoroacetic amide (MSTFA) is a product of Macherey & Nagel, Düren, FRG. Dried pyridine (Merck, Darmstadt, FRG) is stored over molecular sieve 4 Å (Merck). All solvents are of analytical grade or distilled prior to use.

Glassware

A volumetric flask used for the preparation of the uric acid standard solution is calibrated by adjusting the flask to the calibration mark with water at 20 °C and weighing the contents. Taking into regard the density of water at this temperature the flask has a volume of 99.689 ml. For the sampling of serum as well as for dispensing the non-labelled standard solution a mechanised pipettor (Hamilton Microlab P, Bonaduz, Switzerland) equipped with a gas-tight 2.5 ml syringe is calibrated by weighing definite amounts of water at 20 °C. For pipetting of the [1,3-$^{15}N_2$]uric acid standard solution a 25 µl-syringe (SGE, Melbourne, Australia) equipped with a repeating adaptor is used. The precision of the pipetting procedure using the syringe and the Hamilton pipettor is measured by weighing appropriate samples of water ten times at 20 °C. The loss of water during the weighing procedure, due to evaporation, is corrected by weighing the water samples 45 and 90 s after emptying and calculating the original weight at 0 s. The imprecision of the Hamilton pipettor is about 0.03% (coefficient of variation, CV) and of the syringe at 0.3%.

Weighing Procedure

The uric acid certified reference material and the water samples for calibrating the syringe as well as the Hamilton pipettor are weighed with an electronic balance (model 4503, Sartorius, Göttingen, FRG). The volumetric flask is calibrated with the use of a mechanical balance (model 2432, Sartorius, Göttingen, FRG). The accuracy of the balances is ascertained by weighing officially calibrated test weights.

Instruments and Settings

A Finnigan 1020 combined gaschromatograph-mass spectrometer-computer system (Finnigan MAT, Bremen, FRG) equipped with a fused silica capillary column coated with SE-30 (length 25 m, internal diameter 0.32 mm, film thickness 0.1 µm, Macherey & Nagel, Düren, FRG) is employed. The temperature of the column oven is set to 190–240 °C with a programmed rate of 4 °C/min. Helium is used as carrier gas at

an inlet pressure of 35 kPa. Samples are injected by the use of an inlet splitter device, obtained from Gerstel, Mülheim, FRG. The split ratio is set to a value of about 1 : 10; the temperature of the inlet is set to 240°C. During the first 90 s of gas chromatography the reagents injected are sucked off at the end of the column with the use of a divert valve in order to protect the mass spectrometer from excess of solvent. Electron impact ionisation is used for mass spectrometry. The ionisation energy is 70 eV. With the selected ion recording mode the resolution of the mass spectrometer is set to approximately 400 (10% valley definition).

For the recording of mass spectra the instrument is scanned in the range from m/z 50 to 400; the resolution is adjusted to about 800.

For the measurement of radioactivity an LB 282 Linear Analyzer (Berthold, Wildbad, FRG) is used.

Preparation of Standard Solutions

For the preparation of the uric acid standard solution, a specified amount of the certified reference uric acid preparation (purity 99.7%, estimated inaccuracy 0.1%) within the range of 1.9 to 2.1 mg (e. g. 2.056 mg) is dissolved with 0.015 mol/l ammonia solution in the calibrated volumetric flask at 20°C. The weight is multiplied by the factor 0.997 to correct for the impurities as stated in the certificate and by the factor of 0.99689 to correct for the calibrated volume of the flask, thus yielding a concentration of 20.435 µg/ml. This standard solution must be prepared each day that it is needed.

To prepare the [1,3-$^{15}N_2$]uric acid standard solution about 2.5 mg of the labelled uric acid is dissolved in 10 ml 0.015 mol/l ammonia solution using an ultrasonic bath. The labelled working solution is stored in quantities of 1 ml at −20°C. For each individual analytical series a vial of 1 ml of labelled uric acid solution is equilibrated to room temperature.

Preparation of Standards

For the calibration of the mass spectrometer three standards with different concentrations of uric acid are used:

Standard 1 consists of a mixture of 20 µl of the labelled uric acid solution (pipetted with the use of the 25 µl syringe equipped with a repeating adaptor which is fixed to 20 µl) and 225 µl of the non-labelled uric acid standard solution.

Standard 2 comprises 20 µl of the labelled uric acid standard solution and 300 µl of the non-labelled working solution.

Standard 3 is composed of 20 µl labelled and 375 µl non-labelled uric acid solution.

Each of the three different standard mixtures is prepared in triplicate. The 225, 300 and 375 µl aliquots of the non-labelled certified reference material are sampled with the aid of the Hamilton Microlab P-pipettor. The instrument is calibrated prior to use by sampling and weighing appropriate amounts of water 10 times for each volume. The exact volumes were 225.824 µl ± 0.07 µl (s. d.), 300.319 µl ± 0.1 µl (s. d.) and 375.309 µl ± 0.1 µl (s. d.), respectively. From this calibration, it is deduced that standard 1 contains 4.6147 µg, standard 2 6.1370 µg and standard 3 7.6694 µg of the uric acid reference material. Using the aforegoing calibration procedure it is not necessary to know the accurate amount of labelled uric acid; however, care should be taken that equal amounts of the labelled substance are present in the serum samples as well as in the standards. For this purpose the same syringe equipped with the repeating adaptor is used for dispensing the labelled material to samples and standards. The mixtures of labelled and non-labelled uric acid solution are evaporated to dryness in a stream of nitrogen at 60°C.

Sample Preparation

Serum samples containing about 5 µg uric acid (the approximate concentration of uric acid is estimated in a preliminary experiment) are sampled and diluted with water to a final volume of 0.5 ml with the use of the calibrated Hamilton P pipettor. Twenty µl of the [1,3-$^{15}N_2$]uric acid solution are added to the serum samples with the use of the 25 µl syringe. The labelled and non-labelled uric acid is equilibrated by gently shaking the mixture for 30 min at room temperature.

Small columns are prepared by filling the anion exchange resin into Pasteur pipettes to a height of 2.5 cm and washing the material with water. The diluted serum samples spiked with the labelled uric acid are then poured on to the columns. The ion exchange resin is washed with 2 ml water and 0.5 ml acetic acid (12 mol/l). Uric acid is finally eluted from the column with the addition of 1 ml acetic acid (12 mol/l). The eluate is evaporated to dryness in a stream of nitrogen at 80°C. The residue is dissolved in 1 ml methanolic ammonia solution (mixture of 20 ml 25% aqueous ammonia solution and 80 ml methanol). The methanolic solution is decanted into a tapered test tube and evaporated in a stream of nitrogen at 60°C.

The recovery of uric acid after the ion exchange chromatography is about 65% as determined by counting the radioactivity in a sample spiked with ^{14}C-labelled uric acid.

Derivative Formation

The dry residues of standards and samples are reacted with 50 µl of a mixture of MSTFA and pyridine (1 + 1, by vol.). The reaction is carried out at 60°C for at least 12 h.

Gas Chromatography-Mass Spectrometry

Aliquots in the range of 1 to 3 µl of the reaction mixtures are injected into the combined gas chromatograph-mass spectrometer. The quadrupole mass filter of the instrument is scanned over two small mass ranges. The lower mass range is set from −0.25 to +0.25 mass units from the peak center of the molecular ion of the trimethylsilyl derivative of uric acid (m/z 456) and the higher mass range is adjusted between ± 0.25 mass units from the peak center of the trimethylsilyl derivative of the labelled uric acid (m/z 458). The exact position of the peak centers of the molecular ions is measured in a separate run prior to a series of quantitative analyses. During gas chromatography of standards and samples the mass spectrometer repetitively switches between the two mass ranges adjusted. Each mass range is scanned over a period of 0.134 s; a complete recording cycle of the two pre-selected masses takes about 0.280 s. The intensity signals are recorded on a magnetic disk of the computer system. From the intensity data, recorded on the disk, selected ion chromatograms at m/z 456 and 458 may be reconstructed on the computer terminal display for visual inspection. Furthermore the computer quantitates the signals obtained during selected ion recording and prints a report of the peak height and peak area determination at the two m/z-values.

In order to keep the stability of the gas chromatography-mass spectrometry system under continuous control it is important to analyse serum samples and standards alternately.

Calculation Procedure

The concentration of uric acid in the serum samples is calculated from the isotope ratios measured by selected ion recording. Unfortunately, the commercially available ^{15}N-labelled uric acid contains small amounts of non-labelled substance. Therefore, a sample which is free of uric acid still gives rise to an isotope ratio higher than 0. Furthermore, a small amount of naturally

occurring isotopes is present in non-labelled uric acid which contributes to the M + 2 signal of the uric acid derivative. This results in a non-linear relationship between the concentration of uric acid and the isotope ratio measured by selected ion recording. Taking these two facts into account, an equation for the calculation of the results is developed which is described and discussed in detail in the first paper of this series on the determination of cortisol (13).

Accuracy

The accuracy of the method applied here is achieved by using the highly specific technique of selected ion monitoring in combination with the isotope dilution principle which is the most suitable procedure for monitoring recovery.

Precision

The precision of the procedure was determined by measurements on different occasions, of uric acid in control sera used by the Deutsche Gesellschaft für Klinische Chemie for external quality control. For this purpose 5 vials of a control serum were reconstituted with 5 ml distilled water. The contents of the vials were pooled and shaken gently for two hours at room temperature. Then quantities of 1 ml of the reconstituted serum pool were stored in a deep freeze until use. For each measurement occasion one vial of the pool was thawed and allowed to attain room temperature. By applying this procedure the neat method – dependent standard deviation is obtained, thereby excluding any additional vial to vial variability. As shown in table 1 the coefficient of variation is between 0.6 and 1.1%.

Tab. 1. Parameters of precision of the isotope dilution-mass spectrometry method for the determination of uric acid in human serum.

Serum pool	Uric acid (μmol/l)	n	Standard deviation (μmol/l)	Coefficient of variation (%)
281	199.3	6	1.40	0.7
324	217.1	6	1.52	0.7
383	220.1	10	1.76	0.8
279	252.8	6	2.78	1.1
381	274.8	6	3.00	1.1
322	275.4	6	2.75	1.0
280	312.9	6	2.19	0.7
323	320.6	6	3.53	1.1
382	328.9	10	2.30	0.7
276	397.3	8	3.96	1.0
319	400.9	6	3.21	0.8
378	411.0	6	3.29	0.8
380	415.2	10	3.75	0.9
379	424.7	7	4.67	1.1
278	429.4	6	4.30	1.0
321	446.7	6	2.68	0.6
320	499.0	8	3.00	0.6
277	513.9	6	3.10	0.6

Sensitivity

The sensitivity of the selected ion monitoring technique for the detection of uric acid may be estimated from the lower limit of detection of the mass spectrometer. The molecular ion of the uric acid derivative can be detected with a signal to noise ratio of 3 : 1 when a sample of about 10 ng is injected. It should be noted that a 500-fold amount is injected after processing a serum sample.

Discussion

Since it has been generally accepted that high serum levels of uric acid are the root cause of gout, numerous methods for the measurement of uric acid have been developed. These are mainly based on colour reactions with phosphotungstic acid (1), on enzymatic reactions involving uricase (2) and combined reactions with uricase and catalase (4) or aldehyde dehydrogenase (5, 6, 7). As seen from the external quality control surveys of the Deutsche Gesellschaft für Klinische Chemie the results obtained from different methods vary significantly. Therefore, at present several method-dependent target values have to be determined before a collaborative survey is performed and the results of the participants must be evaluated in several method-dependent groups with reference to the different methodological principles and target values. Moreover, different reference values, according to the different methods in use, must be applied in diagnosis and disease control. In view of these drawbacks of the present quality control system in clinical chemistry it may be of great advantage to introduce a reference method, and to use definitive values as target values for external quality control (14). In the present investigation an isotope dilution mass spectrometry procedure for the determination of uric acid was developed thus fulfilling the criteria for a definitive method. Some of the methodological details, such as the formation of the trimethylsilyl derivative and the pre-purification with an ion exchange resin, are similar to the procedure of *Walker* (11) and of *Öhman* (10) who reported the development of a reference method.

A necessary pre-requisite for the development of a definitive method is the availability of the compound to be determined as a primary reference material of known purity. In this case the substance was obtained from the National Bureau of Standards, Washington as a certified reference material. Furthermore, [1,3-$^{15}N_2$]uric acid was commercially available as an isotopically labelled internal standard material suitable for the isotope dilution technique. This substance was

added to the serum samples and equilibrated with the non-labelled uric acid. The labelled and the non-labelled uric acid were then extracted from the serum by the use of an anion exchange resin, a simple chromatographic step by which a number of subtances in human serum e. g. proteins and lipophilic compounds are removed. For gas liquid chromatography the uric acid molecule has to be converted into a stable volatile derivative. As described in the previous report of *Öhman* (10), the trimethylsilyl derivative proved to be most suitable. In order to obtain only one out of several possible isomers, which are probably formed because of various tautomeric forms of the uric acid molecule, it is important to evaporate a solution of the compound before derivative formation in the presence of alkali, preferably methanolic ammonia. The trimethylsilyl derivative then gives a single peak in gas liquid chromatography. The mass spectrum of the compound obtained after electron impact ionisation which is shown in figure 1 reveals a molecular ion at m/z 456 indicating that the derivative contains 4 trimethylsilyl groups. The molecular ion shows high relative intensity which makes it possible to record the uric acid derivative with high sensitivity when the mass spectrometer is adjusted to this m/z-value in the selected ion monitoring mode. In view of this it seemed unnecessary to use more complicated ionisation techniques e. g. chemical ionisation. After processing a serum sample and injecting the trimethylsilyl derivative into the gas chromatography-mass spectrometry system (set at m/z-values 456 and 458) selected ion recordings of the non-labelled and the isotopically labelled uric acid derivatives are obtained. As it may be seen from figure 2, in addition to the two peaks of interest, no further compounds are recorded. This indicates the high specificity of the detection procedure. This high specificity is one of the most important features, which makes it possible to apply the selected ion monitoring technique for the development of a definitive method. Furthermore it appears mandatory to use a purification procedure before mass spectrometry, which offers the highest separation power currently available. Thus, high performance capillary gas liquid chromatography on fused silica, a technique which is superior even to high performance liquid chromatography, was selected as the method of choice. Furthermore it should be noted that a gas liquid capillary column may be easily coupled to the mass spectrometer, whereas complicated interfaces are needed to combine high performance liquid chromatography with mass spectrometry.

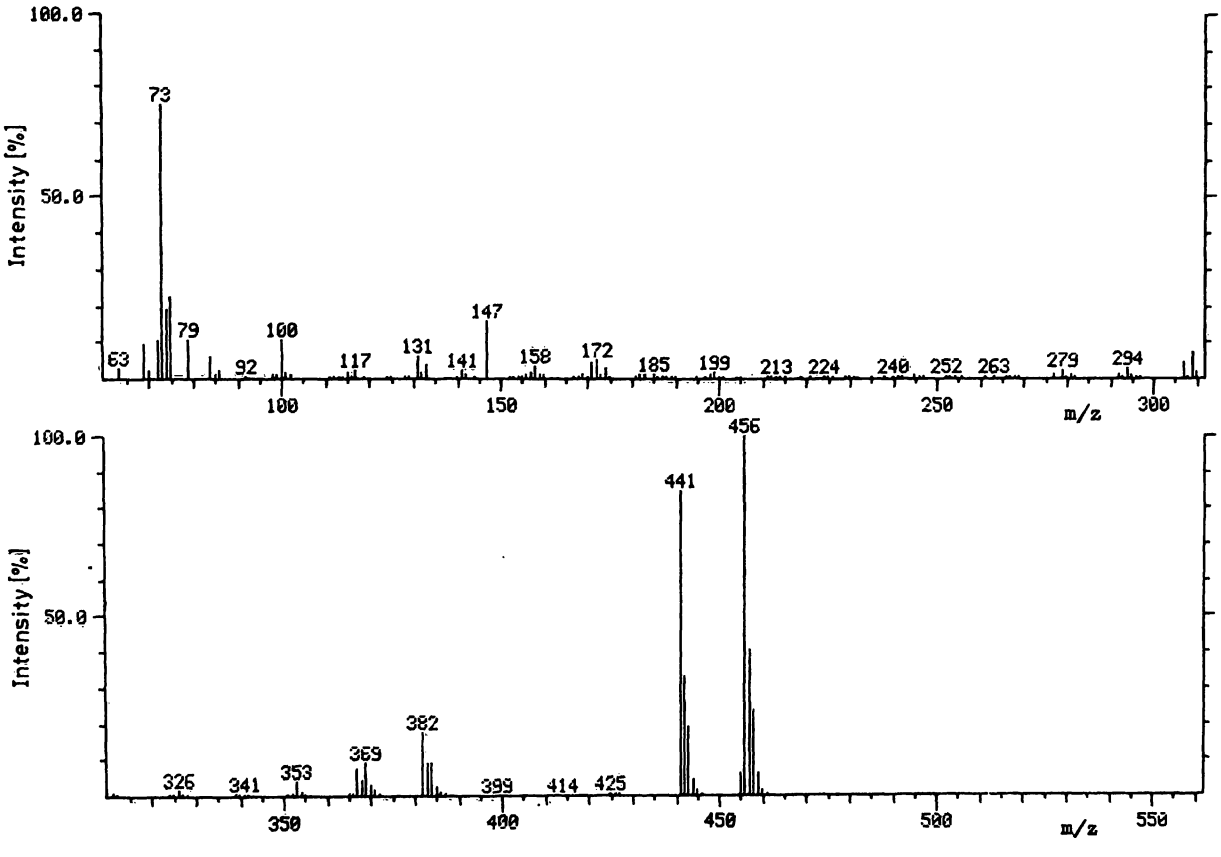

Fig. 1. Mass spectrum (computer print) of the trimethylsilyl derivative of uric acid. For conditions of recording see Instruments and Settings.

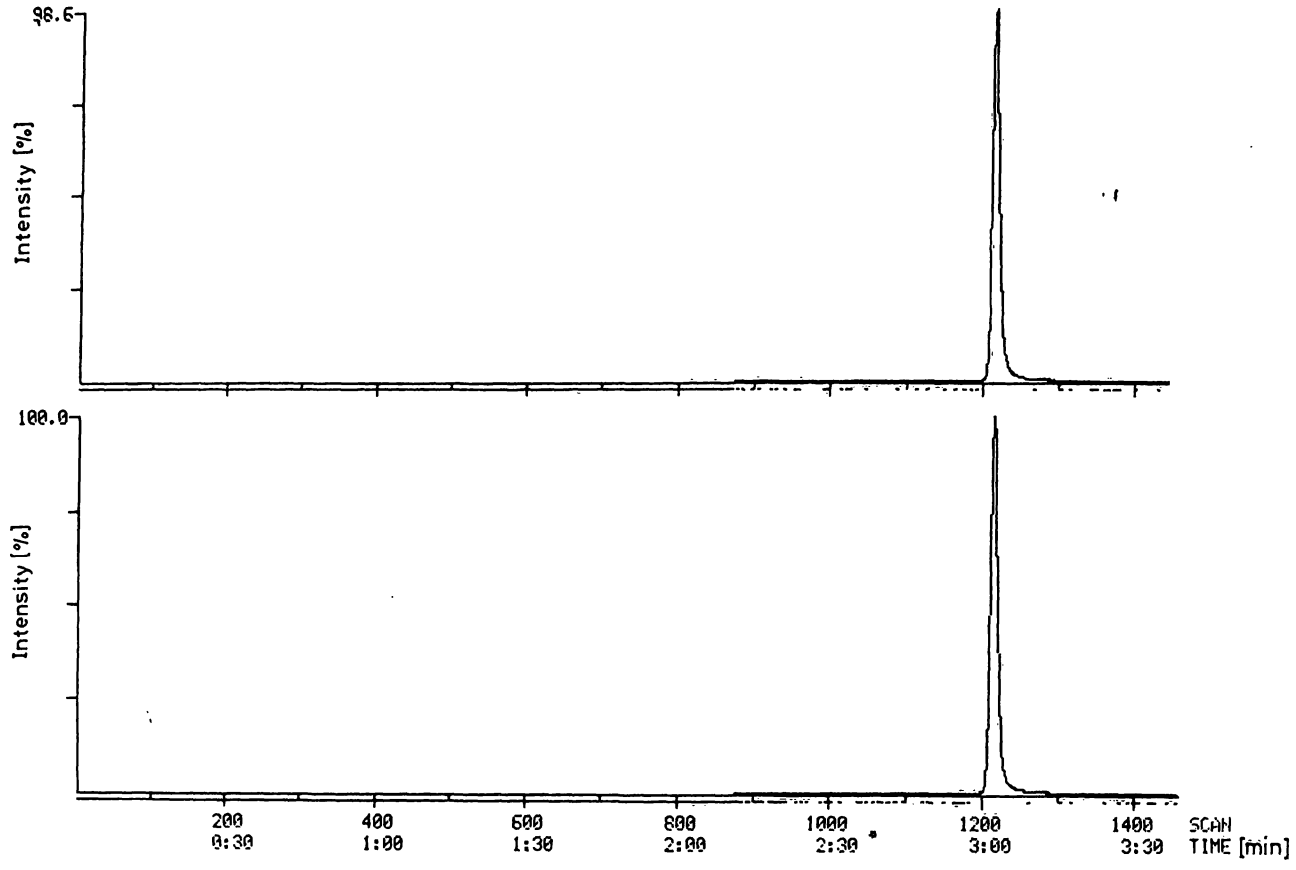

Fig. 2. Selected ion recording (computer print) of the trimethylsilyl derivative of uric acid (upper panel) and of [1,3-$^{15}N_2$]uric acid (lower panel) after processing a serum sample. For experimental details see Materials and Procedure.

For the calculation of the analytical results a set of standards was analysed whose isotope ratios were closely related to those of the serum samples. As it is good analytical practice, standards and samples were injected alternately. The non-labelled uric acid, due to the natural isotope composition of the chemical elements, contributes to a certain extent to the recording of the labelled substance. This results in a non-linear calibration function. Furthermore it should be noted that the labelled uric acid contains a small amount of the non-labelled substance. Taking these facts into consideration, the calculation of the results was carried out by a procedure which is based on well-founded theoretical fundamentals (13, 15) rather than on an empiric calibration curve as described in an earlier investigation (10).

Despite the considerable adavantages of the technique described here there remain some very small sources of unpredictible error in the final results. First, there is an overall imprecision of the method in the range from 0.6 to 1.1% (coefficient of variation from day to day), which is caused by the imprecision of the isotope ratio measurement with the mass spectrometer and the limited precision of pipetting sera and standards. Second, there may be a small systematic error which is due to a possible inaccuracy of the uric acid content in the certified reference material (99.7%) which is less than 0.1% as stated by the National Bureau of Standards.

Apart from these neglegible sources of error the accuracy of a clinical chemical method in general depends on two different criteria of reliability. The first is the specificity of the analytical method. It can be stated that the selected ion monitoring technique as applied here for the measurement of uric acid provides the most specific procedure for the detection of volatile substance in biological fluids. A second crucial point with regard to accuracy of a clinical chemical method is the recovery of a substance during an analytical procedure. Even a highly specific method will produce inaccurate results if the losses of substance cannot be determined accurately. The most reliable procedure for monitoring recovery is the isotope dilution technique. The selected ion monitoring technique provides the most suitable means for monitoring recovery during the analytical procedure. The analytical losses are not only monitored during the pre-instrumental part of the analyses but also

during the final detection process, since the labelled and the non-labelled substance are recorded simultaneously by using the same detection procedure. Although there is no final proof of accuracy for any clinical chemical method, it can be assumed that the procedure reported here, which is based on the isotope dilution principle in combination with the selected ion monitoring technique, provides the most suitable means for the definitive measurement of uric acid in human serum.

The method presented here has been in use for the past two years for the measurement of uric acid in the control sera of the Deutsche Gesellschaft für Klinische Chemie.

Acknowledgement

This work was supported by the Deutsche Gesellschaft für Klinische Chemie and the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen (06/06040/68511). The valuable technical assistance of Miss *Dagmar Mackrodt* is gratefully acknowledged.

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